nature portfolio

Peer Review File

Multi-organ proteomic atlas of obesity regression in male mice

Corresponding Author: Professor Blagoy Blagoev

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Boel et al. describe the results of their efforts to map the proteomic landscape of multiple murine tissues in the condition of diet-induced obesity and define how this landscape changes in association with weight loss. Using DIA-MS proteomics, they profile the proteomes (12K proteins) of 15 tissues from 1) mice with obesity (18 weeks HFD) and then 2, 6, and 12 weeks after the switch back to low-fat diet (leading to weight loss). Age matched controls maintained on the low-fat diet were used as controls for each time point. Notable outcomes include common and tissue-specific protein signatures of obesity and weight loss, along with evidence at the protein level of immune cell/activity retention in weight loss. The results are presented as a publicly available Shiny application, providing a new resource for the field.

Overall, there are many strengths of this manuscript and study that warrant publication. The topic is important for a broad audience and quite timely. The approach is strong (DIA-MS) and the experimental design is appropriate. Nevertheless, there are some addressable concerns regarding the conceptual innovation of the work.

1) As the authors note, most studies of weight loss utilize transcriptomics (bulk and single-cell/nucleus). The novelty here is the information at the protein level, incorporating temporal and multi-organ analysis. The downsides of using mRNA levels to infer function are obvious; however, there is the benefit of sensitivity and cellular resolution with current transcriptomic approaches. Can the authors comment on whether the observed changes in protein levels correlate with available transcriptomic data? Can we use the protein data to infer which cell types could be altered? This seems highly feasible given the adipose datasets that have collectively been generated by the co-authors. There is a broader issue of importance that is not clearly addressed: does the proteomics teach us something that the transcriptomics can/did not?

2) Related to the point above, previous studies have highlighted the retention of phagocytic lipid-associated macrophages and specialized T cells with weight loss (e.g., see work of Hasty lab, Vanderbilt). The proteomics data presented here seems to support those observations (LAMs are Gpnmb+). This part of the paper could be elaborated, incorporating the prior literature (and associated snRNA-seq datasets) to extend this point for the field.

3) The Shiny app is a great addition to the paper and final product. However, it seems limited in its current interface. Users are prompted to pick pathways to visualize. I would assume that many users would also welcome the opportunity to simply query the expression of their favorite protein across tissues and time points. Expanding the capabilities of the web app would increase the likelihood that the study provides a powerful resource for the field.

Minor points:
1. In figure 1a, it would be helpful if the time points for the experiment were included in the digram.

2. In figure 1e, can the scale be split so that the small differences can be observed in the less responsive tissues (e.g. bone, brain, thymus)?

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have conducted proteomic analysis in 15 tissues derived from male C57Bl mice that were obese or following weight-loss at different time points (and age-matched lean mice as 'controls'. The question is interesting, the work is well controlled and appropriately described, and the authors have provided comprehensive datasets. The paper is clearly written and the figures are of good quality and easy to interpret. I further applaud the authors for the development of a web-based tool for use by the scientific community.

The conceptual advance of this work is a better understanding of the change in the proteome of different tissues with obesity and weight loss. Several potential processes were identified, however, no validation experiments were conducted to support possible changes in function/s. Thus, as it stands, the conceptual advance is modest. The study provides interesting leads for future studies.

Concerns / suggestions:

1. The authors conclude that the changes seen in the proteome reflect reversion of obesity. Is it possible that the effects could be mediated by differences in diet composition (i.e. changing from high-fat diet to low-fat diet)? Is this a potential limitation to the interpretation of this work?

2. A major limitation of this study is inferences drawn by measuring the proteome in whole organs. Many tissues are composed of various cell types and drawing tissue wide conclusions without consideration of protein composition in the specific cell types is problematic. For example, in the section titled "Immune remodeling diverges across adipose depots", knowledge of the number and types of immune cells captured across time points would provide a much richer context for discussing immune-related DEPs and interpreting possible implications for adipose tissue functions. I appreciate there is a single line in the discussion addressing this point, but I think a more nuanced discussion is warranted.

3. A second major issue is the absence of confirmatory data to substantiate predicted changes in functions with obesity and regression from obesity. For example, the authors used Reactome Pathway analysis to predict upregulation of SUMO proteins alongside downregulation of Nedd8 proteins, and increased Rho GTPase and RTK signaling accompanied by a slight decrease in second messenger signalling (p.10) in some context (not defined). Key pathways should be interrogated to add depth to this work and definitively identify novel obesity-associated biology.

4. The reason / justification for the analyses from Figure 3f (UpSet plot showing DEPs found in LTR for bone, brain, iBAT, kidney, and pancreas) to Figure 3i is unclear. How and why were these specific tissues grouped for analyses? What is the functional significance of this grouping?

5. For the STRING network analysis in Fig. 3i, it is claimed that this may represent a first molecular signature of past obesity across multiple tissues. Are there existing data repositories of weight loss that the authors could interrogate to test this possibility? Additional analysis in other populations would significantly strengthen the utility of such a predictive 'tool'.

6. Discussion- the authors state that previous studies have largely focused on transcriptomics or single-tissue analyses of diet-induced obesity and its regression. I would like to have seen a deeper analysis / discussion of the similarities of this proteome-level atlas with the existing literature. For example, are transcriptomic changes largely reflective of the proteomic changes?

7. p11/12. "Visceral adipose tissue appears to retain an NF-κB-driven phagocytic response…." – it is very debatable that eWAT is truly visceral adipose tissue – suggest reshaping this paragraph to describe the changes in eWAT and not a broad description of 'visceral adipose'.

8. P.11. "We suspect that these changes may result from a combination of increased mechanical stress, unresolved systemic inflammation, and elevated lipid metabolism .. ". Please carefully define the processes associated with "elevated lipid metabolism" which could mean many things.

9. I found it difficult to read the DEP numbers in many on the figures (e.g. Fig. 3f, 4b and others).

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors perform a comprehensive examination of the proteome of over a dozen tissues at 4 time points during the regression from obesity. In addition to creating a valuable data set deposited on PRIDE, the authors report previously unknown tissue-specific and lasting effects of a high-fat diet on the proteome even after a long-term wash-out on a low-fat diet. The study was well-designed, resource intensive, and well-executed. I imagine it being impactful and of great interest to the readers of Nature Metabolism. I do have the following concerns about the authors' claims and data presentation:

1.) I cannot understand what the authors are trying to communicate with plot in figure 2a. Do the colors represent the tissues as indicated in the plot in Fig. 1a? Is the "Set Size" the number of differentially expressed proteins corresponding to a

tissue? What does "Intersection Size" refer to? What do the colored dots underneath the bar plots mean? Why does the bar plot have a break in the x-axis and gene names written above the axis on the other side of the break?

2.) The y-axes of the plots of 2b are labelled log(FC). Is that log2(FC)? Is the fold change relative to the control mice? Also, some indication of variance and significance for each tissue/time-point should be provided. This comment also applies to similar plots (2c, 3b, 3e, and 3h)

3.) Figure 2: The text on the plots is too small

4.) Figure 3: The text on the plots is too small

5.)The following phrase needs to be supported with data: "Interestingly, the top up-regulated protein in in eWAT in obesity, Gpnmb, becomes further up-regulated during regression of obesity." What statistical test was used to compare the regression of obesity time points with the obesity time points? What was the result of that statistical test?

6.) Figure 3e: The figure, as it is displayed, does not support the following text, "In general, DEPs in these tissues are slightly upregulated in obesity but progressively decreased in expression, reaching significantly reduced levels in long-term regression (Fig. 3e)." Results from statistical significance tests need to be provided. This is related to comment #2.

7.) The following phrase is not supported by the data: "Further examination of the 42 DEPs downregulated in at least two of these tissues (Fig. 3g) showed that they exhibit similar trends in other tissues, despite not reaching statistical significance in all cases (Fig. 3h)." The claim of exhibiting a "similar trend" appears to be based solely on the authors' intuition. A claim cannot be made without a statistical test. Perhaps the authors could use a one-tailed test with the null hypothesis being that the proteins are not down-regulated during regression of obesity.

8.) Why are the down-regulated proteins of eWAT not discussed? It has more down-regulated proteins than any other tissue (Fig. 3d).

9.) A text file key needs to included in the PRIDE repository stating what each file is. There should be a column for the file name, the time point, the tissue, the mouse ID, and the experimental condition (i.e. whether or not it is a control sample).

Reviewer: Nathaniel M. Vacanti

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a nice job addressing the questions/suggestions from reviewers. The ShinyApp is greatly improved and should be a really nice resource for the field.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have done an excellent job at responding to the reviewer's critiques, with the addition of relevant new data, comparative analysis with other data sets, and a suitably modified discussion addressing some of the limitations of this work. I have no further comments and congratulate the authors on pulling together such a comprehensive manuscript.
Reviewed by: Matthew Watt

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed all of my comments.

(Remarks on code availability)

Point-by-point response to the editor and reviewers

We would like to thank the editor and reviewers for their positive view on our work and for their constructive comments and suggestions

Response to editors

Editor:

In the revised manuscript, it will be particularly important to the editors that you address the following concerns:

1] In the revised manuscript, it will be important to integrate and compare your findings with existing literature, in particular transcriptomics datasets and cell type specific variations to determine how well the proteomic changes match previous conclusions. Reviewers 1 and 2 have made clear suggestions in this regard.

Response:

All done, as suggested. We have integrated transcriptomic and single-cell comparisons into the revised manuscript.

Specifically, we directly compared proteomics and matched bulk RNA-seq data from the same cohort (new Supplementary Fig: S5), contextualized persistent eWAT immune signatures using published adipose single-cell atlases (new Supplementary Fig. S11), and added cell-type context for the cross-tissue non-adipose protein module (new Supplementary Fig. S12). The Results and Discussion were revised accordingly. In addition, to support the physiological relevance of discordant extracellular and lipid-associated protein signatures identified by proteomics and not by transcriptomics, we measured circulating cholesterol fractions and observed obesity-associated increases in LDL/VLDL, HDL, and total cholesterol that was normalized after long-term regression (new Extended Data Fig. 1), further illustrating how proteomics captures systemic tissue-milieu remodeling not readily inferred from trancriptomic data alone.

2] Along those lines, it will be important to validate the proposed ‘molecular signature of past obesity across tissues’ using existing literature/data. Reviewer 2 has made suggestions in this regard.

Done.

We compared the cross-tissue protein module identified here with available single-cell and tissue transcriptomic resources and show that many components map to conserved structural RNA metabolism programs rather than a single tissue-restricted pathway (new Supplementary Fig. S12). We also clarify in the Discussion that direct validation in independent multi-organ obesity regression datasets is currently limited by the scarcity of comparable cohorts, and we frame this as a key limitation and direction for future work.

We also validate our own findings from the proteomics data that Hippo signaling is impaired in several tissues/organs (kidney, spleen, bone, iBAT) of the formerly obese mice even after long-term
weight regression (new Extended Data Fig. 2).

3] In light with the comments from the Reviewers, we encourage you to provide some functional validation for at least some key conclusions in the adipose tissue analyses (e.g. using in vitro analyses).

Done.
In addition to the validation of cholesterol abnormalities associated with the obese state of the animal (new Extended Data Fig. 1, described in the comments to point 1 above), we have added orthogonal validation of selected signaling signatures identified from the proteomic atlas. For the adipose tissues, pathway exploration highlighted altered intracellular second messenger/ phosphatidylinositol-associated signaling. We therefore examined PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) by immunohistochemistry showing reduced PIP3 in obese eWAT with only partial recovery after regression (new Extended Data Fig. 3). We further showed that these lower PIP3 levels in the eWAT of the long-term regression animals resulted in reduced AKT activity compared to the corresponding lean controls (new Extended Data Figs. 4).
In addition, iBAT was among the tissues with Hippo pathway-associated changes, validated by Western Blotting with phospho-specific antibodies to the key Hippo signaling players, MOB1 and YAP1 (new Extended Data Fig. 2).

4] Editorially, we also feel that it will be important to provide more in-depth analysis and discussion on the proteomics changes observed in tissues other than adipose tissue (At least 1-2 other tissues) and *if possible* include some functional validation for some key conclusions.
Done.
As described above, we showed and verified decreased Hippo signaling in kidney, spleen, bone and iBAT of the weight regressed animals. As suggested further be reviewers, we have extended both the results and discussion sections by two additional paragraphs each, describing and discussing those findings.

5] Please address the methodological, statistical and data presentation concerns raised by Reviewer 3 and also address and discuss the limitations and caveats highlighted by all the reviewers.
Done. This has now been addressed as suggested.

6] I would like to draw your attention to our trial of a stricter implementation of the Sex and Gender Equity in Research (SAGER) guidelines, which was recently announced (see our Editorial: https://www.nature.com/articles/s42255-022-00581-1). We strongly encourage you to include at least key experiments in male and female mice. If the study is heavily skewed towards one sex, we ask that you indicate this in the abstract, and perhaps even in the title, by adding "...in male mice" or equivalent to the text.
Done.
We have added “in male mice” in both the title and abstract. We also expanded the limitations of discussion, where we previously had a brief mention of this, to now provide more emphasis on the importance of this.

7] Finally, we strongly encourage you to pay special attention to our formatting guidelines, which will be required should the manuscript be accepted for publication at a later stage. As a minimum, please prepare your figures in editable formats, present data as scatter plots, state the number of samples in the figure legends, provide source data for each figure, and provide uncropped scans of all blots and gels. Additionally, please take full advantage of our Extended figures (max. of 10) before making use of Supplementary figures (unlimited). The difference between them is that Extended figures are more easily available to readers, as they appear in the PDF and the

HTML versions of the paper, whereas Supplementary figures need to be downloaded separately. Detailed formatting guidelines can be found here:
https://www.nature.com/natmetab/submission-guidelines/aip-and-formatting
Key points are also summarized in a related Editorial (https://www.nature.com/articles/s42255-023-00841-8).
Thank you for the detailed description. We tried to adhere strictly to the requirements while preparing the revision. We hope we've managed to do that.

Response to Reviewers

Reviewer 1:
Boel et al. describe the results of their efforts to map the proteomic landscape of multiple murine tissues in the condition of diet-induced obesity and define how this landscape changes in association with weight loss. Using DIA-MS proteomics, they profile the proteomes (12K proteins) of 15 tissues from 1) mice with obesity (18 weeks HFD) and then 2, 6, and 12 weeks after the switch back to low-fat diet (leading to weight loss). Age matched controls maintained on the low-fat diet were used as controls for each time point. Notable outcomes include common and tissue-specific protein signatures of obesity and weight loss, along with evidence at the protein level of immune cell/activity retention in weight loss. The results are presented as a publicly available Shiny application, providing a new resource for the field.
Overall, there are many strengths of this manuscript and study that warrant publication. The topic is important for a broad audience and quite timely. The approach is strong (DIA-MS) and the experimental design is appropriate. Nevertheless, there are some addressable concerns regarding the conceptual innovation of the work.

1) As the authors note, most studies of weight loss utilize transcriptomics (bulk and single-cell/nucleus). The novelty here is the information at the protein level, incorporating temporal and multi-organ analysis. The downsides of using mRNA levels to infer function are obvious; however, there is the benefit of sensitivity and cellular resolution with current transcriptomic approaches. Can the authors comment on whether the observed changes in protein levels correlate with available transcriptomic data? Can we use the protein data to infer which cell types could be altered? This seems highly feasible given the adipose datasets that have collectively been generated by the co-authors. There is a broader issue of importance that is not clearly addressed: does the proteomics teach us something that the transcriptomics can/did not?
We generated the bulk transcriptomics data for eWAT and iWAT from the same cohort. We performed a comparative analysis also based on log2FC estimated using linear regression for each timepoint and compared to the protein data. There was only a moderate correlation between protein and mRNA changes with linear model R2 ranged from 0.02-0.46 (new Supplementary Fig. S5a-b). There were also many proteins highly upregulated at the protein level, but inversely showed a downregulation from the transcriptomics data. Pathways enrichment analysis of those proteins identified several upregulated pathways related to transport of small molecules, apoptosis, cellular senescence, histone deacetylation, and fat-soluble vitamin metabolism (new Supplementary Fig. S5c,d).
Notably, several extracellular/secreted and lipid-associated proteins (including Serpina1e and several apolipoproteins) showed opposite-direction regulation at the RNA and protein levels (new Extended Data Fig. 1a,b), highlighting that proteomics complements transcriptomics capturing

biologically relevant changes in tissue protein milieu, including secreted and matrix-associated proteins, that would not have been predicted from RNA-seq alone.
To further assess whether these discordant extracellular and lipid-associated protein changes reflected systemic physiology, we measured circulating cholesterol fractions. Obese mice showed elevated LDL/VLDL, HDL, and total cholesterol levels compared to lean controls, which was mostly normalized in long-term regression (new Extended Data Fig. 1c).
Next, while we don't have the single cell data from this study cohort, we compared our findings to find cellular origin for these discrepancies in another very similar cohort, where snRNA of eWAT was performed (abbreviated epiWAT in their study) – Hinte et al. (Nature, 2024). Here we find that most of the proteins that were upregulated at protein level but discordantly downregulated in bulk RNA, had an enrichment trend towards epithelial and smooth muscle cell populations (new Supplementary Fig. S6)."
All of the above experiments and analyses are described in a new paragraph in Results section (page 6 in the revised manuscript).

To address whether proteomic data can inform on altered cell types, while we don't have single cell data from our study cohort, we provided cell-type contextualization using public single-cell data (Cottam et al., Nature Commun 2022) as well as single-nucleus atlas of eWAT from very similar to our cohort (Hinte et al., Nature 2024). In eWAT, discordant RNA-protein signals mapped preferentially to epithelial and smooth muscle/stromal compartments (new Supplementary Fig. S6), whereas proteins persistently elevated during long-term regression mapped predominantly to lipid-associated macrophage and phagocytic myeloid populations (new Supplementary Fig. S11). We also strived to assess the expression of the cross-tissue module identified from our proteomics data (proteins in the original Fig. 3i). We only found available scRNA data for bone, brain and kidney, where the module genes were broadly expressed across structural and parenchymal cell types rather than restricted to immune populations (new Supplementary Fig. S12). These analyses have also been added to the Results (pages 8-9 in the revised manuscript) and Discussion in the revised manuscript, with limitations mentioning that cell-type attribution is indirect and based on external datasets (page 15).

2) Related to the point above, previous studies have highlighted the retention of phagocytic lipid-associated macrophages and specialized T cells with weight loss (e.g., see work of Hasty lab, Vanderbilt). The proteomics data presented here seems to support those observations (LAMs are Gpnmb+). This part of the paper could be elaborated, incorporating the prior literature (and associated snRNA-seq datasets) to extend this point for the field.
We have implemented this comparison into the paper, comparing the topmost upregulated proteins in LTR of eWAT with expression profiles in existing scRNA-seq datasets, specifically to the Cottam et al. Nat Comm 2022 paper from the Hasty lab, as suggested, as well as the Hinte et al. Nature 2024 paper used for the previous comment (new Supplementary Fig. S11). This has been added to the results sections after discussing Gpnmb, phagocytosis and apoptosis in LTR of eWAT:
"To further resolve the cellular origin of these persistent eWAT signals, we cross-referenced genes corresponding to proteins most strongly upregulated during long-term regression with two published single-cell adipose atlases from weight loss/weight cycling paradigms[6,19]. These datasets show that many of the persistently upregulated proteins, including Gpnmb, Cd68, and Psap, are most highly expressed in lipid-associated macrophages and related phagocytic antigen-presenting myeloid populations (Supplementary Fig. S11). Together with the enrichment

of lysosomal enzymes, Fc receptor signaling, and apoptotic/clearance pathways, this supports the interpretation that long-term regression in eWAT is characterized by persistent macrophage lipid-handling programs, indicating incomplete immune resetting at the tissue protein level."
Furthermore, we also stated the concordance between our findings on Gpnmb and that of the Hasty paper: "This is consistent with prior single-cell studies reporting persistence of Gpnmb+ lipid-associated macrophage states after weight loss (Cottam et al. Nat Comm 2022)."

3) The Shiny app is a great addition to the paper and final product. However, it seems limited in its current interface. Users are prompted to pick pathways to visualize. I would assume that many users would also welcome the opportunity to simply query the expression of their favorite protein across tissues and time points. Expanding the capabilities of the web app would increase the likelihood that the study provides a powerful resource for the field.
Absolutely. The app has now gotten a thorough polish, and now also includes the suggested feature to query the expression of their favorite proteins. The app has also been moved to a more stable server to drastically improve speed and performance. The web domain has also changed to be hosted on a university domain (https://computproteomics.bmb.sdu.dk/app_direct/obesity-atlas/ Username: reviewer | Password: Aa0r7DF8ZHJn2T8).

Minor points:
1. In figure 1a, it would be helpful if the time points for the experiment were included in the digram.
Added.

2. In figure 1e, can the scale be split so that the small differences can be observed in the less responsive tissues (e.g. bone, brain, thymus)?
We wanted to compare different tissues to provide a whole body overview, therefore the axis was kept the same. Instead, I have created the suggested plot and added it to the Supplementary Data Figures and now mentioned it in the main text together with Fig. 1e.

Reviewer 2

The authors have conducted proteomic analysis in 15 tissues derived from male C57Bl mice that were obese or following weight-loss at different time points (and age-matched lean mice as 'controls'. The question is interesting, the work is well controlled and appropriately described, and the authors have provided comprehensive datasets. The paper is clearly written and the figures are of good quality and easy to interpret. I further applaud the authors for the development of a web-based tool for use by the scientific community.

The conceptual advance of this work is a better understanding of the change in the proteome of different tissues with obesity and weight loss. Several potential processes were identified, however, no validation experiments were conducted to support possible changes in function/s. Thus, as it stands, the conceptual advance is modest. The study provides interesting leads for future studies.

Concerns / suggestions:

1. The authors conclude that the changes seen in the proteome reflect reversion of obesity. Is it possible that the effects could be mediated by differences in diet composition (i.e. changing from high-fat diet to low-fat diet)? Is this a potential limitation to the interpretation of this work?

This a good point, which we agree should have been discussed more extensively. The weight loss is induced by a diet switch from HFD to LFD, which imposes both a drastic reduction in calorie intake and a change in macronutrients. It is therefore likely that the changes in the proteome in different tissues induced by the diet switch are not just caused by the reduced obesity per se but also by the decrease in calorie intake (impacting e.g., insulin levels) and the change in macronutrients. In fact, the early effects of the diet switch are like to be mainly the result of the reduced calorie intake and the change in diet. To minimize the effects of micronutrients, the LFD was selected to match HFD. We have now made sure to emphasize that the changes in proteome is the result of reduced obesity as well as the diet and calorie changes.

Added text to the discussion: "However, weight loss in this model was induced by switching from a HFD to a LFD, which simultaneously reduces caloric intake and alters macronutrient composition. Consequently, some of the proteomic changes observed during regression may reflect not only reduced adiposity but also metabolic adaptations to decreased caloric intake and dietary composition. Similar considerations apply to most diet-induced obesity paradigms where weight loss is achieved through diet reversal. To minimize confounding effects of micronutrients, the LFD was selected to match the micronutrient composition of the HFD."

And added this statement to the limitations: "Some of the proteomic changes between obese and lean mice are likely to have resulted from the diet change, rather than weight loss per se."

2. A major limitation of this study is inferences drawn by measuring the proteome in whole organs. Many tissues are composed of various cell types and drawing tissue wide conclusions without consideration of protein composition in the specific cell types is problematic. For example, in the section titled "Immune remodeling diverges across adipose depots", knowledge of the number and types of immune cells captured across time points would provide a much richer context for discussing immune-related DEPs and interpreting possible implications for adipose tissue functions. I appreciate there is a single line in the discussion addressing this point, but I think a more nuanced discussion is warranted.

We agree that tissue-level proteomics limits direct cell-type attribution. We therefore expanded the initial single line in the discussion to the following:

“Finally, an important limitation of our study is that proteomic profiling was performed on whole tissues and therefore reflects a composite signal of multiple cell types. Consequently, changes in protein abundance may arise from altered cell-type composition, shifts in cellular activation states, or both. While we leveraged published single-cell and -nucleus adipose atlases to infer likely cellular contributors to selected signatures, these inferences are indirect and depend on external datasets generated under related but not identical experimental conditions. Therefore, our data define tissue-level remodeling, whereas precise cell-type attribution and spatial context will require future studies integrating spatial proteomics or matched single-cell approaches.”
Additionally, also in relation to point 2 from reviewer 1 on the same topic, we have also done comparisons of the proteomics data with public single cell datasets (new Supplementary Fig. S11). This addition provided cell-type context for the immune-related DEPs discussed in eWAT, particularly the lysosomal, Fc receptor, and clearance-associated proteins enriched during long-term regression. Please see response to point 2 of reviewer 1 above as well.

3. A second major issue is the absence of confirmatory data to substantiate predicted changes in functions with obesity and regression from obesity. For example, the authors used Reactome Pathway analysis to predict upregulation of SUMO proteins alongside downregulation of Nedd8 proteins, and increased Rho GTPase and RTK signaling accompanied by a slight decrease in second messenger signalling (p.10) in some context (not defined). Key pathways should be interrogated to add depth to this work and definitively identify novel obesity-associated biology.
We agree that pathway-level enrichment should not be interpreted without mechanistic context. We have now clarified the temporal and tissue context of the reported Reactome enrichments and revised the text to emphasize that these analyses primarily illustrate how the web tool can be used for hypothesis generation.
Importantly, we had already expanded the interpretation of the neddylation pathway. Nedd8 regulates cullin-RING E3 ubiquitin ligases, which mediate the majority of ubiquitin conjugation reactions. The observed reduction in Nedd8 provided a mechanistic rationale to interrogate ubiquitin signaling further. We therefore performed ubiquitin-site occupancy analysis and proteasome quantification, revealing coordinated proteostasis alterations in iBAT (Fig. 4e,f,g). These additions strengthen the biological interpretation of the pathway analysis while acknowledging that further functional validation of RTK/Rho remains.
Last paragraph of the results has been changed to: “This tool integrates our differential expression analysis results with Reactome pathway data (downloaded April 28, 2025), enabling users to explore any pathway of interest and extract system-wide regulatory patterns beyond those discussed in this manuscript, as well as explore protein expression profiles based on user input. As an illustration of its utility, we examined selected pathways and observed coordinated regulation of SUMOylation and neddylation proteins (Supplementary Fig. S17), as well as changes in Rho GTPase-, RTK-, and intracellular second messenger-associated proteins (Supplementary Fig. S21)”
And this text was added to the beginning of the ubiquitin-related analyses: “Consistent with this pattern, pathway-level analysis using our Shiny-based webtool also indicate reduced abundance of proteins involved in neddylation (Supplementary Fig. S17), a post-translational modification that activates cullin-RING E3 ligases, the largest family of E3 ubiquitin ligases and major mediators of cellular ubiquitination (Nguyen et al., 2017).”
Finally, we followed up on the intracellular second messenger signature identified through the webtool. As several of the underlying components pointed toward phosphatidylinositol-related signaling in WAT, we performed PIP3 staining and Western blotting for the PIP3-dependent AKT

kinase in eWAT, and the results supported altered PIP3/AKT signaling during obesity and regression (new Extended Data Figs. 3-4).

4. The reason / justification for the analyses from Figure 3f (UpSet plot showing DEPs found in LTR for bone, brain, iBAT, kidney, and pancreas) to Figure 3i is unclear. How and why were these specific tissues grouped for analyses? What is the functional significance of this grouping?
We thank the reviewer for the comment and have now clarified this rationale in the revised text. In long-term regression, kidney, bone, and brain showed the largest number of downregulated DEPs in non-adipose tissues, and also displayed a considerable overlap in downregulated DEPs (Fig. 3d,f). We therefore focused our downstream analysis on this subset to determine whether a cross-tissue signature of long-term regression could be identified. This analysis revealed a coherent group of LIM-domain and RNA metabolism related proteins (Fig. 3g), which also exhibited similar expression profiles in other tissues (Fig. 3h) and formed a functionally interconnected network of proteins based on STRING analysis (Fig. 3i). Together, identify a potential multi-organ molecular signature of past obesity that was otherwise not apparent when examining the tissues individually.

5. For the STRING network analysis in Fig. 3i, it is claimed that this may represent a first molecular signature of past obesity across multiple tissues. Are there existing data repositories of weight loss that the authors could interrogate to test this possibility? Additional analysis in other populations would significantly strengthen the utility of such a predictive 'tool'.
We again agree with the reviewer that the broader relevance of the proposed multi-organ signature should be interrogated further. We were unable to identify directly comparable multi-organ obesity-regression datasets spanning these tissues.
However, to provide at least some cellular context, we used the UCSC Cell Browser (Speir et al., Bioinformatics 2021), to explore three (non-obesity) single cell atlases – the only publicly available single-cell atlases we could find: bone (Perrin et al., Elife 2024), brain (Zhao et al., BioRxiv 2019), and kidney (Miao et al. Nature Communications 2021). The protein module showed broad enrichment across structural and stromal cell compartments rather than being confined to immune populations (new Supplementary Fig. S12). We also put more emphasis on this point, clarifying that independent (non-adipose) weight loss datasets are currently scarce, while acknowledging the need for future cross-cohort validation, with the following paragraph added to the discussion: "Importantly, these cross-tissue changes indicate that long-term effects of obesity are not restricted to adipose tissue. Consistent with this interpretation, the genes comprising this module are expressed at detectable levels across multiple structural and parenchymal cell compartments in independent single-cell atlases of bone, brain, and kidney, indicating that the protein module reflects a broadly conserved cellular machinery rather than tissue-specific artifacts. This may represent a molecular signature of past obesity shared across multiple tissues, that is not conventionally captured in obesity research. Direct validation in independent cohorts is currently limited by the scarcity of comparable datasets spanning multiple non-adipose organs and regression of obesity. These findings extend the concept of 'obesity memory' beyond immune and adipose compartments to include fundamental cellular homeostasis pathways in diverse organs."
Finally, we further examined the module of proteins depicted in Fig. 3i, which were identified by the proteomics analyses as potential molecular signature of past obesity across multiple tissues. Since many of these proteins are LIM-domain containing proteins linked to the Hippo signaling pathway, we assessed the total protein levels and the activation-associated phosphorylation status of key components of the Hippo pathway, namely MOB1 and Yap1, by Western blotting.

While the decline of MOB1 and Yap1 expression during obesity regression varied across the examined bone, iBAT, kidney and spleen tissues, their phosphorylation status clearly indicated highly reduced activities in the tissues of the regressed cohort compared to the mice that were never obese (new Extended Data Fig. 2).

6. Discussion- the authors state that previous studies have largely focused on transcriptomics or single-tissue analyses of diet-induced obesity and its regression. I would like to have seen a deeper analysis / discussion of the similarities of this proteome-level atlas with the existing literature. For example, are transcriptomic changes largely reflective of the proteomic changes?
We have now added bulk RNA-seq for this cohort as well as comparisons to existing public snRNA-seq datasets (new Supplementary Fig. S5; new Fig. S6; new Extended Data Fig. 1). Please see response to point 1 of reviewer 1 above. In addition, we have added the following text to the discussion in the revised manuscript:
“A key advance of the present study is the proteome-level resolution of tissue remodeling. While transcriptomic analyses have provided invaluable cell-type and pathway insights, RNA abundance does not necessarily predict protein levels, particularly for secreted, extracellular, and long-lived structural proteins. In our dataset, RNA-protein concordance was modest, and several proteins displayed opposite directionality, including Serpina1e and Apoc3. These patterns likely reflect translational regulation, differential protein stability, and accumulation of circulating or matrix-associated proteins within tissues. Proteomics therefore captures aspects of the tissue protein milieu that are not reliably inferred from transcript abundance. Consistent with this, proteins displaying RNA-protein discordance were enriched for pathways related to extracellular transport, stress response, chromatin regulation, and apoptosis, suggesting that post-transcriptional regulation preferentially affects tissue-remodeling and stress-associated processes.”

7. p11/12. “Visceral adipose tissue appears to retain an NF-κB-driven phagocytic response….” – it is very debatable that eWAT is truly visceral adipose tissue – suggest reshaping this paragraph to describe the changes in eWAT and not a broad description of ‘visceral adipose’.
We thank the reviewer for pointing it out - this has now been changed as suggested.

8. P.11. “We suspect that these changes may result from a combination of increased mechanical stress, unresolved systemic inflammation, and elevated lipid metabolism .. “. Please carefully define the processes associated with “elevated lipid metabolism” which could mean many things.
We use the parent terms from Reactome, but in the sub-categories for ‘lipid metabolism’, the largest affected group is’ fatty acid metabolism’. We have changed the main text from “elevated lipid metabolism” to “alterations in fatty acid metabolism”.

9. I found it difficult to read the DEP numbers in many on the figures (e.g. Fig. 3f, 4b and others).
We apologize for the small font size. This has now been adjusted to follow the journal’s guidelines.

Reviewer 3

The authors perform a comprehensive examination of the proteome of over a dozen tissues at 4 time points during the regression from obesity. In addition to creating a valuable data set deposited on PRIDE, the authors report previously unknown tissue-specific and lasting effects of a high-fat diet on the proteome even after a long-term wash-out on a low-fat diet. The study was well-designed, resource intensive, and well-executed. I imagine it being impactful and of great interest to the readers of Nature Metabolism. I do have the following concerns about the authors' claims and data presentation:

1.) I cannot understand what the authors are trying to communicate with plot in figure 2a. Do the colors represent the tissues as indicated in the plot in Fig. 1a? Is the "Set Size" the number of differentially expressed proteins corresponding to a tissue? What does "Intersection Size" refer to? What do the colored dots underneath the bar plots mean? Why does the bar plot have a break in the x-axis and gene names written above the axis on the other side of the break?

We have modified the plot slightly to improve clarity for readers not familiar with UpSet plots, specifically:

We changed the naming in the figure from “Set Size” to “Total proteins per tissue” and “Intersection Size” to “Shared proteins across tissues”.

We also tweaked the figure legend to explain in more detail: “a, UpSet plot showing all significantly DEPs across timepoints for each tissue. Each color corresponds to a tissue, consistent with the color scheme used in other Figures (such as Fig. 1a and 2b). Horizontal bars indicate the total number of DEPs identified in each tissue, and vertical bars represent the number of DEPs shared among the connected tissues (intersect) shown by filled circles below. The UpSet plot was modified to show both the largest intersections (left) and the DEPs shared across the most tissues (right), separated by the X-axis break.”

2.) The y-axes of the plots of 2b are labelled log(FC). Is that log2(FC)? Is the fold change relative to the control mice? Also, some indication of variance and significance for each tissue/time-point should be provided. This comment also applies to similar plots (2c, 3b, 3e, and 3h)

We thank the reviewer for pointing out that. It was log2 fold change relative to age-matched lean control mice. This is now specified in the plots and figure legends. We also added the standard errors to the plots. All values for the standard errors along with calculations of significance for each tissue/timepoint can be found in Supplementary Table 22.

3.) Figure 2: The text on the plots is too small

Fixed now to adhere to journal guidelines. Also found a similar font mistake in Figure 4 that has now been changed as well to adhere to journal guidelines, thanks to this comment.

4.) Figure 3: The text on the plots is too small

Fixed as well now to adhere to journal guidelines.

5.)The following phrase needs to be supported with data: "Interestingly, the top up-regulated protein in in eWAT in obesity, Gpnmb, becomes further up-regulated during regression of obesity." What statistical test was used to compare the regression of obesity time points with the obesity time points? What was the result of that statistical test?

We thank the reviewer for this comment as well. We performed the contrast: (RegressionW12 – LeanW12) – (RegressionW0 – LeanW0) = 0. This resulted in Log2FC = +1.82, t = 1.54, p-value

= 0.14, ie non-significant trend. We have revised the sentence to: “Interestingly, the top upregulated protein in eWAT in obesity, Gpnmb, trended to become further upregulated during regression of obesity after adjusting for age-related effects (log2FC = 1.82, p-value = 0.14)”

6.) Figure 3e: The figure, as it is displayed, does not support the following text, "In general, DEPs in these tissues are slightly upregulated in obesity but progressively decreased in expression, reaching significantly reduced levels in long-term regression (Fig. 3e)." Results from statistical significance tests need to be provided. This is related to comment #2.
Supplementary Table 22 contains results from all statistical testing. We also adjusted the text slightly to better reflect what is statistically significant. It now reads: “Analyzing the expression profiles for downregulated DEPs in these tissues, we found a tendency for them to be slightly upregulated in obesity but then progressively decrease with regression of obesity, eventually reaching statistically significant reduced levels in long-term regression compared to age-matched lean controls (Fig. 3e).”

7.) The following phrase is not supported by the data: "Further examination of the 42 DEPs downregulated in at least two of these tissues (Fig. 3g) showed that they exhibit similar trends in other tissues, despite not reaching statistical significance in all cases (Fig. 3h)." The claim of exhibiting a "similar trend" appears to be based solely on the authors' intuition. A claim cannot be made without a statistical test. Perhaps the authors could use a one-tailed test with the null hypothesis being that the proteins are not down-regulated during regression of obesity.
The reviewer is correct, this is purely based on qualitative observations and not statistical testing. We have now performed a one-tailed test on the temporal slopes of log2 fold changes across regression timepoints, which directly evaluates directional trends and addresses the reviewer’s concern.
Results section: “Further examination of the 42 overlapping DEPs from bone, brain, and kidney, showed that they exhibit a similar trend also in spleen, thymus, and iBAT (median per-protein temporal slope in $\log_2$ fold-change versus age-matched lean < 0; one-sided t-test p-values < 10^{-8}; Fig. 3h), even where individual time points did not reach statistical significance.”

Methods section: “Temporal trends for Fig. 3h were assessed by regressing log2 fold-change versus age-matched lean controls on ordered timepoints (OBE, STR, MTR, LTR) and testing whether per-protein slopes were < 0 using one-sided one-sample t-tests.”
Here are the exact results of the statistical test for the reviewer to assess:

Tissue	n	median_slope	mean_slope	p_t_test	p_wilcox
Bone	40	-0.198	-0.223	1.41e-16	9.09e-13
Brain	39	-0.0923	-0.0896	3.64e-11	3.64e-12
Kidney	41	-0.216	-0.237	2.36e-16	4.55e-13
Spleen	41	-0.182	-0.187	1.95e-16	3.18e-12
Thymus	40	-0.0683	-0.0671	8.64e-10	1.08e-7
iBAT	38	-0.139	-0.183	9.25e-9	8.77e-8

8.) Why are the down-regulated proteins of eWAT not discussed? It has more down-regulated proteins than any other tissue (Fig. 3d).
To maintain focus in Fig. 3 we only emphasized non-adipose tissues, we then go on to discuss eWAT downregulation together with the other adipose depots separately in Fig. 4.
For improved clarity about this separation we changed the text to the following: “Investigating the significantly downregulated proteins in long-term regression, we unexpectedly found kidney,

bone, and brain among the most affected tissues, closely following eWAT which will be discussed together with other adipose depots later (Fig. 3d).”

9.) A text file key needs to included in the PRIDE repository stating what each file is. There should be a column for the file name, the time point, the tissue, the mouse ID, and the experimental condition (i.e. whether or not it is a control sample).
Thank you for noticing. The requested table has now been added.

Point-by-point response to the remaining reviewer comments

1] Please provide the login details (username and password) for the webtool in the Data Availability Statement, if you cannot make it fully accessible as of yet. There seems to be conflicting information provided in different sections of the checklist. As of now the link requires a username and password so this should be included in the DAS.
Added temporary login details for the webtool in the Data Availability Statement.

2] A summary sentence might be useful at the end of the Results section as it currently feels like it ends a bit abruptly
Added a summary sentence to the final paragraph of the Results section:
“Overall, these findings illustrate how our obesity atlas and accompanying webtool can help facilitate hypothesis generation and enable exploration of tissue-specific and system-wide adaptions during obesity and its regression.”

3] Please provide catalog numbers for animal models, diets etc within the methods section
Added catalog number for animals.

4] Please provide antibody dilutions in the methods section
Specified antibody dilutions for the western blotting experiments.

5] We do not allow 'online methods' and supplementary methods so please confirm that all methodological information pertaining to this manuscript is available in the 'Methods' section included therein
All methodological information pertaining to this manuscript is available in the Methods section and the heading has now been renamed from ‘Online Methods’ to ‘Methods’.

6] Please include a statistical analysis section for all analyses conducted on non-omics data (E.g. the WBs)
Added a separate section for the analysis of non-omics data.

7]PXD066875 is currently not accessible. Please fix this. Previous works have been able to make these data available without the need for an affiliated DOI.
This issue has been resolved, and the dataset is now publicly accessible.

8] Please upload your extended data figures as "Figure" rather than "Supplementary" files.
Thanks, we’ll do that.